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The Dominant Source of $CD4^+$ and $CD8^+$ T-Cell Activation in HIV Infection Is Antigenic Stimulation

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Summary: To distinguish between antigenic stimulation and $CD4^+$ T-cell homeostasis as the cause of T-cell hyperactivation in HIV infection, we studied T-cell activation in 47 patients before and during highly active antiretroviral therapy (HAART). We show that expression of human leukocyte antigen (HLA)-DR, CD38, and Ki67 on T cells decreased during HAART but remained elevated over normal values until week 48 of therapy. We confirm previous reports that T-cell activation correlates positively with plasma HIV RNA levels (suggesting antigenic stimulation), and negatively with CD4 count (suggesting $CD4^+$ T-cell homeostasis). However, these correlations may be spurious, because misleading, due to the well-established negative correlation between CD4 count and plasma HIV RNA levels. To resolve this conflict, we computed partial correlation coefficients. Correcting for CD4 counts, we show that plasma HIV RNA levels contributed to T-cell hyperactivation. Correcting for plasma HIV RNA levels, we show that $CD4^+$ T-cell depletion contributed to T-cell activation. Correcting for both, activation of $CD4^+$ and $CD8^+$ T cells remained positively correlated. Because this suggests that $CD4^+$ and $CD8^+$ T-cell activation is caused by a common additional factor, we conclude that antigenic stimulation by HIV or other (opportunistic) infections is the most parsimonious explanation for T-cell activation in HIV infection. Persistence of HIV antigens may explain why T-cell activation fails to revert to levels found in healthy individuals after 48 weeks of therapy. **Key Words:** Activation—Proliferation—T lymphocytes—CD4—CD8—Antiretroviral therapy.

T lymphocytes of HIV-infected people have increased expression of activation markers human leukocyte antigen (HLA)-DR and CD38 (1–11) and increased proliferation rates. The latter has been demonstrated using two different techniques. First, by determining the fraction of dividing cells through expression of the nuclear antigen Ki67 (12), it was shown that T-cell proliferation rate is increased maximally twofold to threefold in the $CD4^+$ population, and sixfold to sevenfold in the CD8 population (13–16). This limited increase in the division rate is consistent with results of studies that measured the replicative history of T cells by the average telomere lengths (17,18). The second technique, using deuterated glucose to label DNA in vivo, showed that the turnover of $CD4^+$ and $CD8^+$ T cells in HIV-infected patients is about three times higher than that of uninfected individuals (19). Increased turnover of $CD4^+$ and $CD8^+$ T lymphocytes has also been observed in macaques infected with simian immunodeficiency virus using BrdU to label DNA in vivo (20).

Two models have been proposed to explain the hyperactivation and increased proliferation of T cells in HIV-1 infection. One model contends that T-lymphocyte acti-

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vation in HIV infection is driven by antigens from HIV and/or from other pathogens (15,21,22). Alternatively, increased production of $CD4^+$ T cells may be a homeostatic response to compensate for the loss of $CD4^+$ T cells that are killed by HIV (23,24). The goal of this study was to determine which of these two mechanisms best explains the T-lymphocyte activation in HIV infection. We therefore performed cross-sectional and longitudinal analyses of the activation status of T lymphocytes, T-lymphocyte population density, and plasma HIV RNA levels in a large cohort of HIV-infected patients before and during HAART.

MATERIALS AND METHODS

Study Population

The activation and proliferation status of T cells was analyzed in 47 patients from the previously described CHEESE study cohort (25) with a sustained plasma HIV RNA response to levels <50 copies/ml. Briefly, this is a randomized study comparing antiviral efficacy of zidovudine (Retrovir) plus lamivudine (Epivir) plus saquinavir-soft-gelatin-capsules (SQV-SGC, Fortovase) versus zidovudine plus lamivudine plus indinavir (Crixivan) in HIV-1–infected patients. Antiretroviral-naive patients were eligible for study treatment if at the moment of screening plasma HIV RNA levels were at least 10,000 copies/ml and/or if CD4 counts were <500 cells/ml and/or if they had a history of HIV-related symptoms (U.S. Centers for Disease Control and Prevention [CDC] stage B or C). During 48 weeks of treatment, the virologic and the CD4 count responses were no different between the two treatment arms (data not shown). Of the selected patients, 25 were from the indinavir arm and 22 from the SQV-SGC arm.

Healthy Controls

As controls for the expression of $Ki67^+$ on T lymphocytes, cryopreserved peripheral blood mononuclear cells (PBMCs) from 5 HIV-seronegative blood bank donors were used. As controls for expression of CD38 and HLA-DR on T cells, freshly isolated PBMCs from 12 healthy HIV-seronegative donors were used.

Blood Sampling

Blood samples were obtained at baseline, and every 4 weeks through week 24, and every 8 weeks from week 24 through week 48 of treatment.

Plasma Viral Load

Plasma HIV RNA levels were measured using an investigational version of the ultra-sensitive quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay (Amplicor HIV-1 Monitor, Roche Diagnostic Systems, Neuilly, France). The lower limit of detection was 50 copies/ml.

Monoclonal Antibodies

Peridinin chlorophyll protein (PerCP)-labeled CD4, PerCP-labeled CD8 and phycoerythrin (PE)-labeled HLA-DR monoclonal antibodies were obtained from Becton Dickinson (San Jose, CA, U.S.A.). Fluorescein isothiocyanate (FITC)-labeled CD38 and FITC-labeled Ki67 monoclonal antibodies were obtained from Immunotech (Marseilles, France).

Flow Cytometry

The fraction of activated $CD4^+$ and $CD8^+$ T cells was determined by three-color fluorescence-activated cell sorter (FACS) analysis using monoclonal antibodies against CD4 (or CD8), CD38, and HLA-DR on heparin-anticoagulated venous blood (FACScan; Becton Dickinson Immunocytometry Systems). In 16 patients, the fraction of proliferating T cells was determined before therapy and at weeks 4, 12, 24, and 48 of highly active antiretroviral therapy (HAART) by measuring the expression of the nuclear antigen Ki67 on cryopreserved PBMCs.

Statistical Analysis

The nonparametric Mann-Whitney-U Test (Wilcoxon Rank-Sum W tests) was used to compare patients with controls. Longitudinal changes of patient characteristics were tested using the nonparametric Wilcoxon matched pairs signed-rank test. Pearson's correlation coefficients were computed to measure bivariate correlations. Partial correlations were calculated to analyze the correlation that remains between two variables after removing the correlation that is due to their mutual association with a third variable. Correlations were computed for pooled data of all timepoints (weeks 0–48) and for data of baseline only. Similar correlations were found for baseline and for the pooled data, although the p values were generally higher in the baseline correlations probably due to a smaller sample size (Tables 1 to 3). Reported p values are two sided. All statistical analyses were performed using SPSS for Windows, release 8.0.0 (Chicago, IL, U.S.A.) Nonlinear regression analysis was performed using Mathematica, version 2.1 (Wolfram Research, Inc., Champaign, IL, U.S.A.).

RESULTS

Expression of Activation Markers Before Highly Active Antiretroviral Therapy

T cells expressing Ki67 were considered to be proliferating. Ki67 is a protein expressed by cells in the late G1 and the S, G2 and M phase of the cell cycle (12). T cells expressing HLA-DR were considered to be activated cells. The $CD8^+$ T cells expressing CD38 were also to be considered activated (2,4,6–11). We confirm previous reports (1–11) that, before beginning HAART, the expression of HLA-DR and Ki67 on $CD4^+$ T lymphocytes, and the expression of HLA-DR, CD38, and Ki67 on $CD8^+$ T lymphocytes, is higher in HIV-1 infected patients compared with healthy controls (Fig. 1).

TABLE 1. *Correlation between plasma viral load and T-cell activation/proliferation*

Activation marker	All data		Baseline data	
	r	Controlled for CD4	r	Controlled for CD4
%HLA-DR (CD4)[a]	0.22[b]	0.20[c]	0.46[c]	0.33[c]
%Ki67 (CD4)[d]	0.47[b]	0.46[b]	0.39[c]	0.44; $p = .1$
%HLA-DR (CD8)[a]	0.27[b]	0.26[b]	0.20; $p = .2$	0.23; $p = .1$
%CD38 (CD8)[a]	0.64[b]	0.60[b]	0.64[b]	0.56[b]
%Ki67 (CD8)[d]	0.47[b]	0.54[b]	0.45; $p = .07$	0.51[c]

[a] $n = 461$ for pooled data; $n = 47$ for baseline data.
[b] $p < .001$.
[c] $p < .05$.
[d] $n = 79$ for pooled data; $n = 16$ for baseline data.
HLA, human leukocyte antigen.

Effect of Highly Active Antiretroviral Therapy on Plasma Viral Load, CD4 Count, and CD8 Count

The median plasma viral load decreased from 40,000 copies/ml to <50 copies/ml in 16 weeks ($p < .001$). The CD4 count increased from 301 ± 28 at baseline to 507 ± 40 cells/ml at week 48 ($p < .001$). The increase of CD4 count during the first 4 weeks of therapy was higher (2.0 cells/mm^3/day) compared with the mean CD4 count rise during later 4-week intervals (0.38 cells/mm^3/day), in agreement with a biphasic response pattern of the CD4$^+$ T cells to HAART (26). The CD8 count decreased from 1050 ± 70 cells/mm^3 at baseline to 870 ± 60 cells/mm^3 at week 48 ($p = .023$).

Effect of Highly Active Antiretroviral Therapy on Expression of HLA-DR, CD38, and Ki67 Cells on T Lymphocytes

Expression of all activation markers on CD4$^+$ and CD8$^+$ T cells gradually decreased during HAART (all p values < .005). At week 48, however, the mean expression levels were still significantly higher than in healthy controls, even though all patients had plasma HIV RNA levels below 50 copies/ml for a median period of 32 weeks (range, 0–44 weeks; Fig. 1), which is consistent with the findings of others (27–31).

Correlation Between CD4 Count, Plasma Viral Load, and Percentage of Activated and Proliferating T Cells

Previous studies have concluded that T-cell activation markers are positively correlated with the plasma viral load, and negatively with CD4 counts (11,14). For all three activation markers, we report similar correlations (Tables 1 and 2). However, these correlations may be spurious, because of the indirect effect of the negative correlation between CD4 count and plasma HIV RNA levels (at baseline $r = -0.4$; $p = .04$). We therefore corrected for the negative correlation between CD4 count and plasma HIV RNA by computing partial correlations. The positive correlations that were observed between the plasma viral load and the expression of activation markers on T lymphocytes (Table 1) are scarcely affected by controlling for the indirect effect of the CD4 count. Apparently, independent of homeostatic effects through the CD4 count, the plasma HIV RNA level has a true contribution to the T-cell hyperactivation. This

TABLE 2. *Correlation between CD4 count and T-cell activation/proliferation*

Activation marker	All data		Baseline data	
	r	Controlled for viral load	r	Controlled for viral load
%HLA-DR (CD4)[a]	–0.46[b]	–0.43[b]	–0.50[b]	–0.40[c]
%Ki67 (CD4)[d]	–0.58[b]	–0.55[b]	–0.60[c]	–0.64[c]
%HLA-DR (CD8)[a]	–0.12[c]	–0.03; $p = .3$	–0.13; $p = .4$	–0.2; $p = .2$
%CD38 (CD8)[a]	–0.42[b]	–0.32[b]	–0.53[c]	–0.39[c]
%Ki67 (CD8)[d]	–0.30[c]	–0.12; $p = .3$	–0.2; $p = .4$	–0.3; $p = .2$

[a] $n = 461$ for pooled data, $n = 47$ for baseline data.
[b] $p < .001$.
[c] $p < .05$.
[d] $n = 79$ for pooled data, $n = 16$ for baseline data.

TABLE 3. *Correlation between activation in CD4+ and CD8+ subsets*

Activation markers		All data				Baseline data			
CB8	CD4	r	Controlled for CD4	Controlled for viral load	Controlled for CD4 and VL	r	Controlled for CD4	Controlled for viral load	Controlled for CD4 and VL
%HLA-DR[a]	%HLA-DR	0.58[b]	0.62[b]	0.56[b]	0.62[b]	0.36[c]	0.49[b]	0.32[c]	0.45[b]
%CD38[a]	%HLA-DR	0.39[b]	0.24[b]	0.33[b]	0.23[b]	0.46[b]	0.26[c]	0.24[c]	0.15[c]
%Ki67[d]	%Ki67	0.62[b]	0.49[b]	0.28[b]	0.32[c]	0.21; $p = .45$	0.38; $p = .18$	0.10; $p = .8$	0.22; $p = .4$

[a] $n = 461$ for pooled data, $n = 47$ for baseline data.
[b] $p < .001$.
[c] $p < .05$.
[d] $n = 79$ for pooled data; $n = 16$ for baseline data.
VL, viral load; HLA, human leukocyte antigen.

supports the model that antigenic stimulation plays a role in T-cell hyperactivation during HIV infection. Similarly, the negative correlation between the CD4 count and activation of $CD4^+$ T cells persists after controlling for the plasma HIV RNA load. This suggests a true additional role for CD4 homeostatic effects on $CD4^+$ T-cell activation. Thus, in the $CD4^+$ T-cell compartment, both mechanisms seem to play a role. The negative correlation between the CD8 activation markers and the CD4 count however largely disappears (HLA-DR, Ki67, Table 2) when controlling for plasma HIV RNA. Apparently, activation of $CD8^+$ T cells largely results from plasma HIV RNA levels and is not directly related to the CD4 count.

In contrast to the inverse relationship between CD4 count and the percentage of activated $CD4^+$ T cells, which suggests homeostasis (Table 2), no association was observed between the CD8 count and percentage of $CD8^+$ T cells expressing HLA-DR or CD38 ($r = 0.12$ and $r = -0.01$, respectively; $p > .05$). This seems reasonable because homeostasis is not expected to play a role in the expanded CD8 population. A weak positive correlation between CD8 count, and the percentage of Ki67-expressing CD8 cells was observed ($r = 0.22$; $p = .047$).

Rate of Decay of T-Cell Activation Markers During Highly Active Antiretroviral Therapy

To determine whether a relationship exists between decreasing plasma HIV RNA levels and T-cell activation during HAART, we estimated the second phase slope (weeks 4–48) of HIV RNA from plasma for each patient by linear regression analysis. In addition, the decay rates of the activation markers on T cells were estimated assuming that the percentage of activated T cells at baseline is a, and that T-cell activation decreases with rate per day c to a level of healthy individuals b. We estimated a, b, and c by fitting equation $y = b + a[\exp(-ct)]$ to the measurements of T-cell activation, where y is the percentage of activated T cells and t is time. No significant correlations were found between the rate of decline of plasma HIV RNA and the decay rates c of HLA-DR and Ki67 expression on $CD4^+$ T cells, or the decay rates of HLA-DR, CD38, and Ki67 expression on $CD8^+$ T cells (each absolute Pearson's coefficient <0.37, each p value > .24). These findings indicate that the daily decrease of plasma HIV RNA plays a limited role in the decay rate of T-cell activation.

To determine the influence of increasing CD4 counts on $CD4^+$ T-cell deactivation during HAART, we also estimated the daily increase of the CD4 count during HAART for each patient. Because of the biphasic pattern of CD4 count increase, the speed of increase was estimated for the first phase (weeks 0–4), and the second phase (weeks 4–48), using linear regression analysis. During both phases, no significant correlations were observed between the daily increase in CD4 count and the decay rates of expression of HLA-DR and Ki67 on $CD4^+$ T cells (each absolute Pearson's coefficient <0.24, each p value > .45). These findings indicate that the daily increase of the CD4 count plays a limited role in the decay rate of T-cell activation.

Correlation Between Activation and Proliferation Status of $CD4^+$ and $CD8^+$ Cells

We confirm observations by Sachsenberg et al. (14) that $Ki67^+$ expression on $CD4^+$ and $CD8^+$ T cells is positively correlated (Table 3, Fig. 2*D*).Similarly, the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells was positively correlated with the percentage of $HLA\text{-}DR^+$ or $CD38^+$ $CD8^+$ T cells (Fig. 2*C*). This suggests that $CD4^+$ and $CD8^+$ T-cell activation is driven by a common mechanism.

That the positive correlation between $CD4^+$ and $CD8^+$ T-cell activation persists after controlling for the indirect

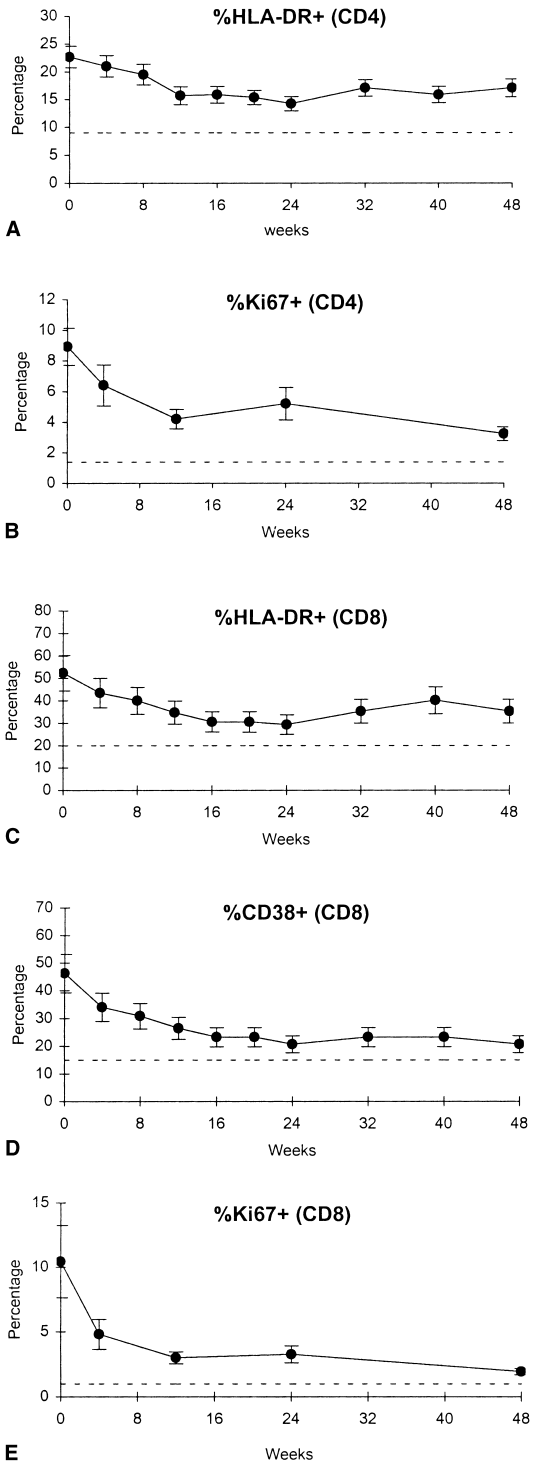


FIG. 1. The effect of highly active antiretroviral therapy (HAART) on expression of activation antigens on T lymphocytes. Mean values are shown. *Bars* represent standard error of the mean. *Dotted lines* indicate mean expression levels in healthy HIV-seronegative controls. (**A, B**) Expression of human leukocyte antigen (HLA)-DR and Ki67 on $CD4^+$ T cells, respectively. (**C–E**) Expression of HLA-DR, CD38, and Ki67 on $CD8^+$ T cells, respectively. At week 48, mean expression levels of the activation markers on T cells were still significantly higher than in healthy controls ($p < .05$ each comparison of patients versus controls [Mann-Whitney U-test]).

effect of CD4 count (Table 3) suggests that other factors than CD4 homeostasis drive $CD4^+$ and $CD8^+$ T-cell activation. This positive correlation, however, also persists when we control for plasma HIV RNA level, and when we control for both CD4 count and plasma HIV RNA levels. These findings indicate that additional factors may play a role in T-cell activation, such as immune activation by other infections or HIV antigens that are not correlated with the plasma HIV RNA load.

DISCUSSION

The aim of this study was to determine the mechanisms involved in increased activation and division of T lymphocytes in HIV-infected patients. We found a negative correlation between the CD4 count and the percentage of activated $CD4^+$ T cells, which remains after controlling for plasma HIV RNA load. Observations like this suggest a homeostatic response of the $CD4^+$ T-cell population to compensate for the $CD4^+$ T-cell depletion in HIV infection (23,24). Several other observations, however, argue against a general role for homeostasis in the increased activation of $CD4^+$ and $CD8^+$ T cells in HIV infection. First, the activation and proliferation are also resent in the expanded $CD8^+$ T-cell population. Second, because the expression of activation markers on $CD4^+$ and $CD8^+$ T cells remains positively correlated after controlling for the CD4 count (Table 3), factors other than $CD4^+$ T-cell depletion appear to play a role in driving the activation of both $CD4^+$ and $CD8^+$ T cells. Third, the percentage of $CD4^+$ T cells expressing HLA-DR and Ki67 decreased rapidly after the start of HAART even though $CD4^+$ T cells were still depleted (15). Fourth, at no timepoint during therapy, the decrease in the expression of HLA-DR and $Ki67^+$ on $CD4^+$ T cells was correlated to the increase in CD4 count (data not shown).

The plasma viral load correlated positively with the expression of HLA-DR, CD38, and Ki67 on T cells. This positive correlation suggests that plasma HIV RNA load and HIV replication drive T-cell activation. However, two of our observations suggest that additional factors play a role. First, even though at week 48 of HAART, all patients had plasma HIV RNA loads below 50 copies/ml for a mean interval of 32 weeks, the level of T-cell activation and proliferation remained significantly higher than in healthy controls. Second, the decay rate of the percentage of activated and proliferating T cells was not correlated with the elimination rate of HIV RNA from plasma. Third, the expression of activation markers on $CD4^+$ and $CD8^+$ T cells remained positively correlated after controlling for plasma HIV RNA, suggesting that

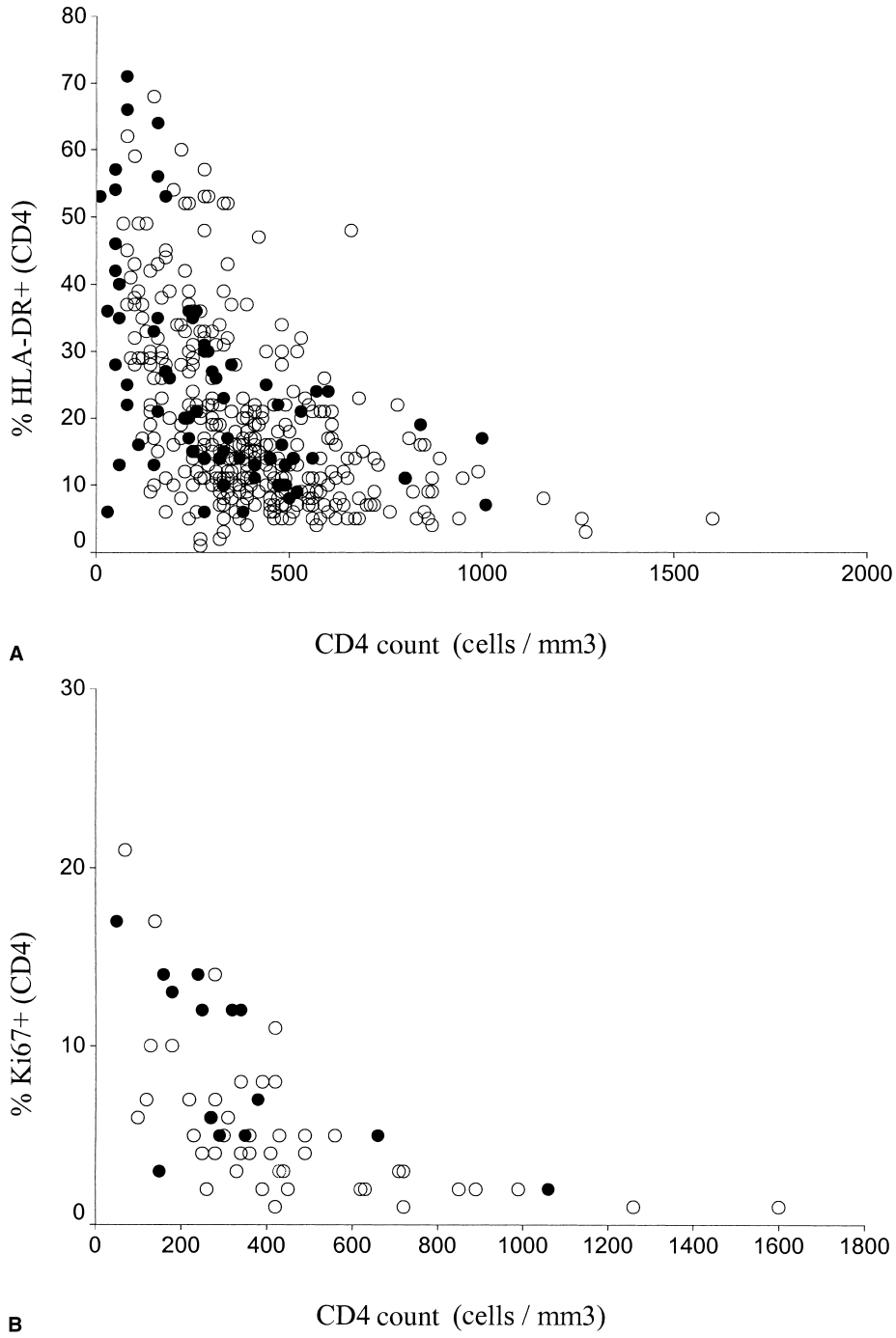
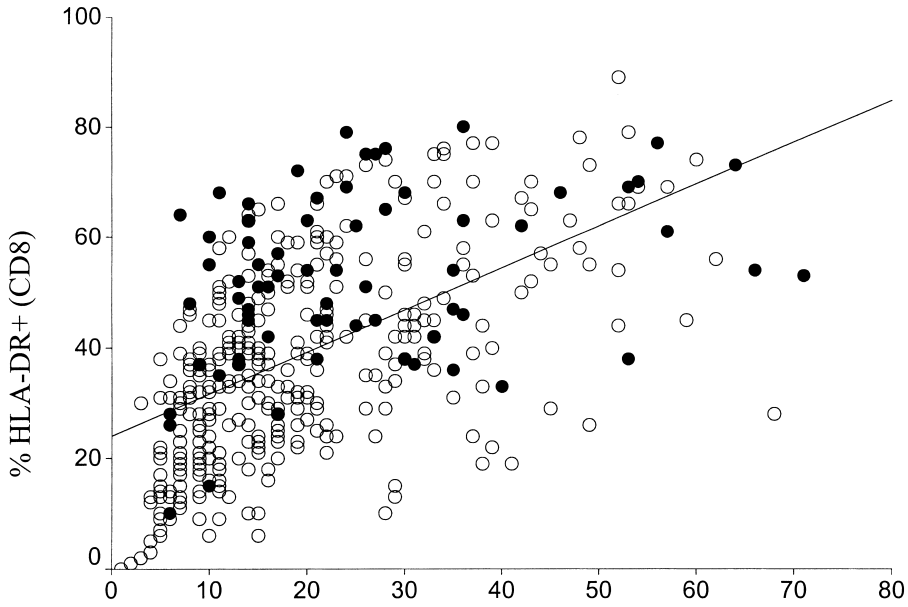


FIG. 2. (**A, B**) Negative correlation between CD4 count and expression of human leukocyte antigen (HLA)-DR and Ki67 on $CD4^+$ T cells, respectively, suggesting homeostatic effects in the $CD4^+$ T-cell population. *Black dots* represent baseline data; *white dots* represent data from week 4 through week 48 of therapy. (**C**) Correlation between expression of HLA-DR on $CD4^+$ T cells and on $CD8^+$ T cells. (**D**) Correlation between expression of Ki67 on $CD4^+$ T cells and on $CD8^+$ T cells. (**A, C:** n = 461; (**B, D:** n = 79.

other factors than plasma HIV RNA contributes to T-cell activation.

Which additional factors, apart from CD4 homeostasis and plasma HIV RNA levels, could contribute to T-cell activation? The positive correlation between fractions of activated cells in the $CD4^+$ and $CD8^+$ T-cell population, which persist after controlling for the CD4 count and the plasma HIV viral load, suggests that $CD4^+$ and $CD8^+$ T-cell activation are governed by similar factors. Thus, we believe that the most parsimonious explanation for the hyperactivation of both $CD4^+$ and $CD8^+$ T-cell populations is antigenic stimulation. This may involve (long-lived) antigens from HIV and/or other (opportunistic) pathogens.



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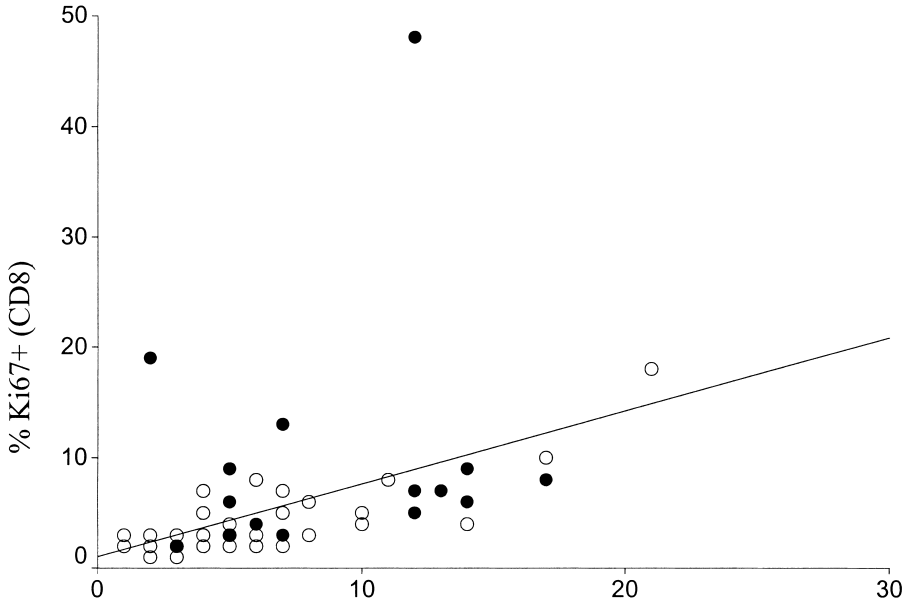

D

FIG. 2. Continued.

The negative correlation between the CD4 count and T-cell activation, which could be taken as evidence for a homeostatic response of the $CD4^+$ T-cell population, may also be explained by antigenic stimulation. A low CD4 count increases the risk of developing opportunistic infections with *Pneumocystis carinii,* cytomegalovirus (CMV), or *Mycobacterium avium* complex (32,33). Moreover, in the blood of patients with low CD4 counts signs of active CMV and Epstein-Barr virus replication have been observed (34–37). These (opportunistic) infections are associated with increased T-cell activation (11,38–40). Antigenic stimulation also explains the positive correlation between the plasma viral load and T-cell activation. A high plasma viral load is associated with an

increases the risk of developing opportunistic infections, and replication of HIV itself also increases antigenic load.

The slow decay of T-cell activation during HAART may be explained in two ways. First, the clearance of antigens from other anatomic compartments than the blood, for instance, lymphoid tissue, is expected to be slow (41–43). In line with this, we observed persistence of HIV p24 antigen in lymphoid tissue, after 18 months of HAART with plasma viral loads below 50 copies/ml (data not shown). Secondly, low-level ongoing HIV replication during HAART may play a role. Based on theoretical considerations (44) and the detection of HIV mRNA in lymphoid tissue of patients on HAART with plasma viral load <50 copies/ml (45,46), it has been hypothesized that a low level of HIV replication may occur during HAART. In addition, it has been demonstrated that the presence of episomal HIV-1 infection intermediates persist in patients with undetectable plasma HIV RNA levels during HAART (47).

A strong interaction exists between HIV replication and T-cell activation because productive HIV infection is largely restricted to $CD4^+$ T cells that are activated (22,48). Several predator-prey type mathematical models of HIV infection describe this interaction, assuming that activated $CD4^+$ T cells are the primary target cells of HIV (48). In contrast to our observations (Fig. 1) however, the number of activated $CD4^+$ T cells increases during HAART in these models. Thus, the number of target cells increases if HIV is suppressed. Our results therefore suggest that current mathematical models should be extended with mechanisms for CD4 T cell activation by HIV and/or other antigens. One such mechanism, obviously, would be to allow for the immune response to HIV (and/or other antigens).

In conclusion, our results suggest that antigenic stimulation is the dominant mechanism of T-cell activation in HIV infection, rather than $CD4^+$ T-cell homeostasis. Persistence of HIV antigens, or low-level ongoing HIV replication during HAART may explain why T-cell activation fails to revert to levels of healthy individuals after 48 weeks of therapy.

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